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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

The research we have conducted over the third year of this research program is contained in this report. We have made substantial progress towards completing all of the proposed research in this program within proposed time frame i.e. final report on 9/29/12. During the last year, we have demonstrated that 1) decreases in neural activity in the BLA and hippocampus are critical for SPS-enhanced fear renewal (specific aims #1a and b), 2) have linked SPS-enhanced glucocorticoid receptor expression to SPS-induced extinction recall deficits (specific aim #1c), 3) demonstrated that the BLA is critical for social interactions (specific aim #2), and 4) demonstrated that SPS induces cognitive deficits, which may be relevant to emotional regulation (specific aim #3).

**15. SUBJECT TERMS**
PTSD, SPS, anxiety, fear, conditioning, prefrontal cortex, hippocampus, amygdala

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## Introduction

In this third year annual report to the Department of Defense (DoD), we present the findings that have been obtained during the third year of our research program (9/30/10 – 9/29/11). We previously demonstrated that single prolonged stress (SPS) induces extinction recall deficits, enhances fear renewal, and increases glucocorticoid receptor (GR) expression in the hippocampus and prefrontal cortex (PFC). Based on these findings, we set two major goals for our third year of research. 1) Identify neural regions critical for mediating SPS-induced extinction recall deficits and SPS-enhanced fear renewal and 2) determine if SPS-enhanced GR expression is linked to SPS-induced extinction recall deficits. Significant progress had been made in accomplishing these two goals, over the last year.

In addition we aimed to determine the role of the basolateral complex of the amygdala (BLA) in mediating social interactions (specific aim #2). The BLA is critical for expression of fear and anxiety (Sotres-Bayon, Bush et al. 2004). As a result, we predicted that inactivation of the BLA would reduce anxiety in the social interaction test, and thus enhance social interactions. Contrary to our expectations, BLA inactivation decreased social interactions, which suggests that the BLA does have an important role in mediating social processes, but in a way that is different from what we had hypothesized. We plan to examine the relevance of these finding to disrupted social processes in post traumatic stress disorder (PTSD), using our SPS animal model.

Finally, an important part of our specific aim #3 was to demonstrate that SPS exposure also disrupted emotional regulation. SPS-induced extinction recall deficits suggest SPS rats are compromised in using extinction memories to reduce fear levels. Similarly, SPS-enhanced fear renewal suggests SPS rats are comprised in using contextual processing to regulate fear levels. We planned to continue exploring potential mechanisms by which SPS exposure might result in emotional regulation deficits. In order to do this, we conducted a study that examined the effects of SPS on a cognitive process relevant to emotional regulation, using set-shifting paradigm. Using an established protocol to classify error types (Floresco et al, 2008), we demonstrated that SPS increases never-reinforced errors during set-shifting, suggesting that SPS disrupts specific aspects of cognitive flexibility. We will continue to explore the neural mechanisms leading to these these findings.

We have made significant progress toward achieving our specific aims during the last year of research in this program, and are well situated to finishing this research program by 9/30/12.

## Body

Below, in the Key Accomplishments section, we list the four key research findings obtained during the last year. These are: 1) SPS-enhanced fear renewal is mediated by decreases in neural activity in the hippocampus and BLA, 2) SPS-enhanced GR expression in the hippocampus and PFC contributes to SPS-induced extinction recall deficits, 3) inactivation of the BLA attenuates social interactions, and 4) SPS increases the frequency of never-reinforced error during set-shifting. we present these key findings. A full description of methods and statistics that we employed can be found in the Supporting Data section.

## Key Research Accomplishments

**1) SPS-enhanced fear renewal is mediated by decreases in neural activity in the hippocampus and BLA.**

In this study, we aimed to identify brain regions that are critical for mediating SPS-induced extinction recall deficits and SPS-enhanced fear renewal (specific aims #1a & b). To examine this, we conducted fear conditioning, extinction, extinction recall, and renewal tests and then measured c-fos expression in regions of the medial prefrontal cortex (mpfc, infralimbic (IL) region, prelimbic (PL) region), hippocampus, BLA, and locus coeruleus. C-Fos is an immediate early gene that is upregulated with increased neural activity. We initially proposed to examine the effects of SPS on neural activity using single unit electrophysiology, however c-Fos has the advantage in that one can examine neural activity in multiple brain regions simultaneously. We currently have results for SPS-induced changes in c-Fos expression during extinction recall and renewal. The experimental design is illustrated in Figure 1a. We did not find difference in c-fos expression between SPS and control rats during extinction recall (Figure 1c), but we did find decreased c-Fos expression in the BLA and hippocampus of SPS rats during fear renewal (Figure 1c). This suggests that SPS-enhances fear renewal by decreasing neural activity in the BLA and hippocampus.

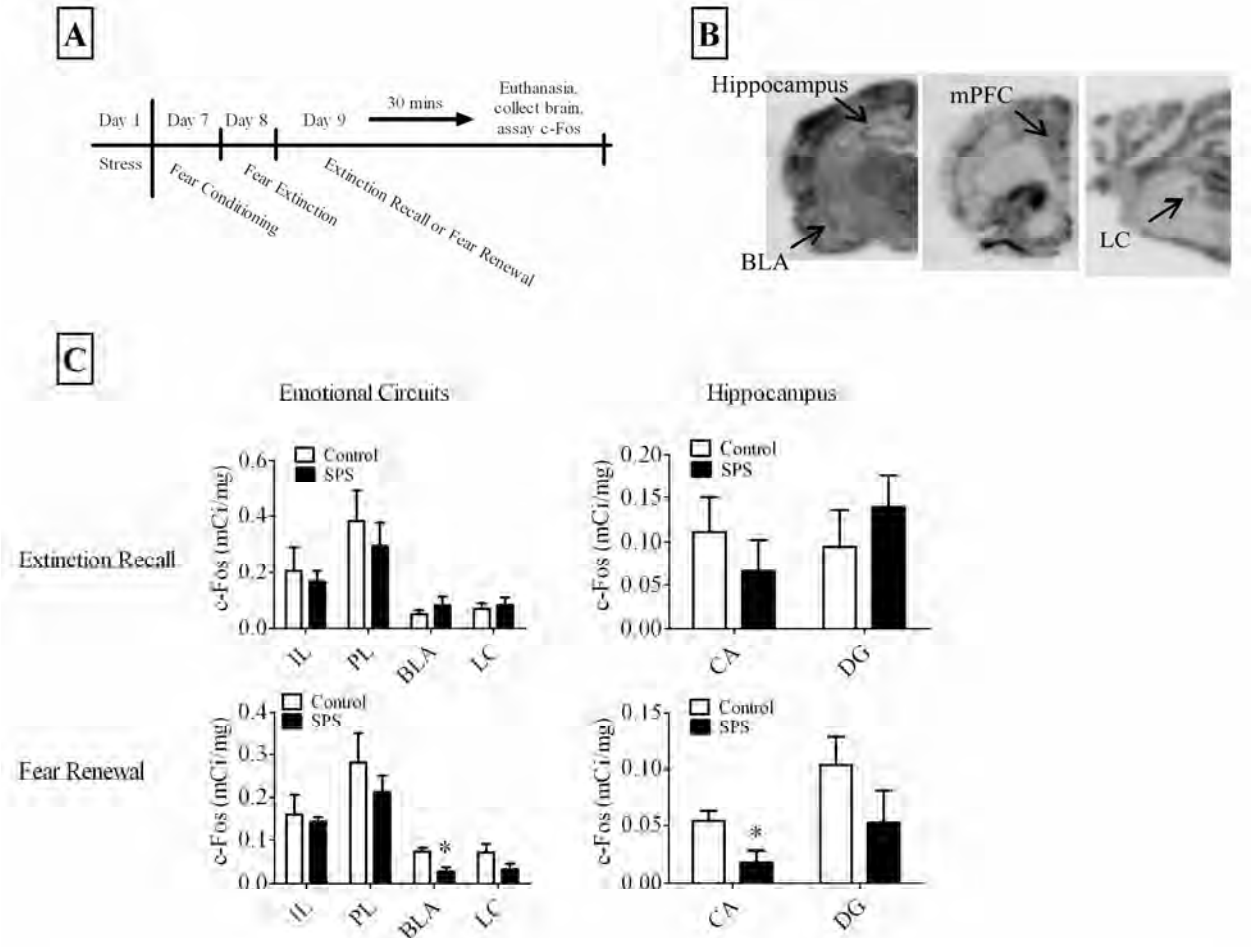


**Figure 1.** A) Experimental design used for the study. B) Illustration of image used to quantify c-Fos expression. Higher c-Fos levels correspond to darker regions on the image. C) Results. SPS had no effect on c-Fos expression during extinction recall, but decreased c-Fos expression in the BLA and CA regions of the hippocampus during fear renewal. IL – infralimbic cortex, PL – prelimbic cortex, BLA – basolateral complex of the amygdala, LC – locus coeruleus, DG – dentate gyrus.

**2) SPS-enhanced GR expression in the hippocampus and PFC contributes to SPS-induced extinction recall deficits**

One of our main goals in the third year of research was to determine relationship between SPS-enhanced GR expression and SPS-induced extinction recall deficits (Specific Aim #1c). In order to do this, we systematically reduced the number of stressors that comprise SPS from three stressors to one stressor and observed the effects this reduction in SPS intensity had on extinction recall. If SPS-enhanced GR expression and SPS-induced extinction recall deficits are linked, then reducing the intensity of SPS should simultaneously reduce stress-enhanced GR expression and stress-induced extinction recall deficits. The algorithm used to select stressors is illustrated in Figure 2. The general experimental design is illustrated in Figure 3. Figures 4 – 9 demonstrate that reducing the number of stressors that comprise SPS eliminates stress-induced extinction recall deficits. Figure 9 shows that reducing the intensity of SPS reduces stress-enhanced GR expression in the hippocampus and PFC, but did not eliminate these effects. These results suggest that SPS-enhanced GR expression in the hippocampus and PFC contribute to SPS-induced extinction recall deficits.

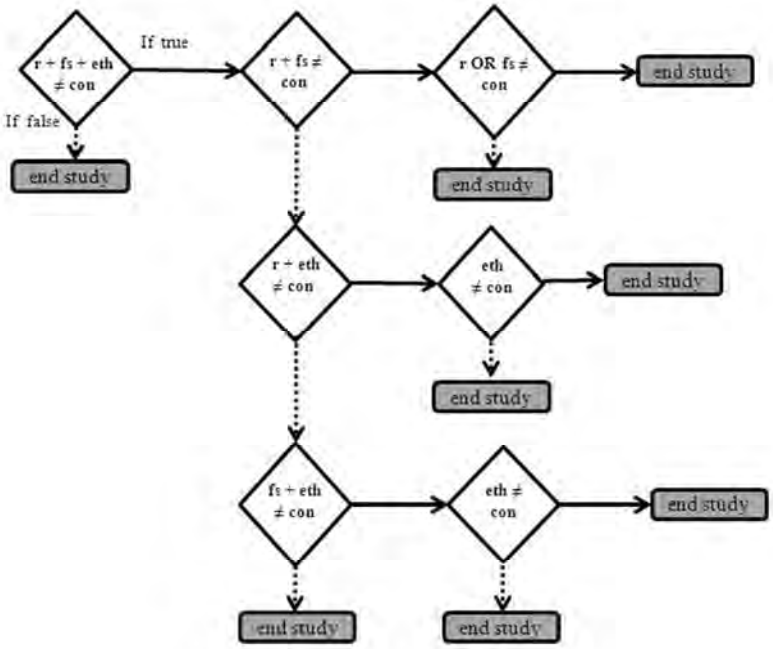


**Figure 2.** Algorithm used to systematically reduce the number of stressors that comprise SPS, and by doing so systematically reduce the intensity of SPS.

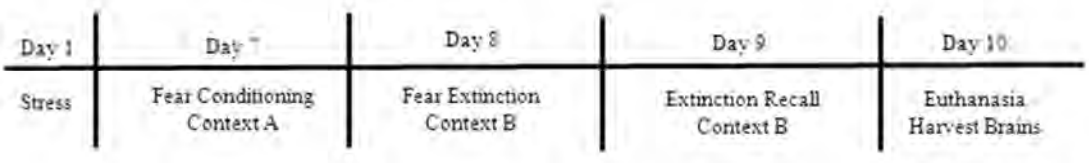


**Figure 3.** Experimental design used in this study.

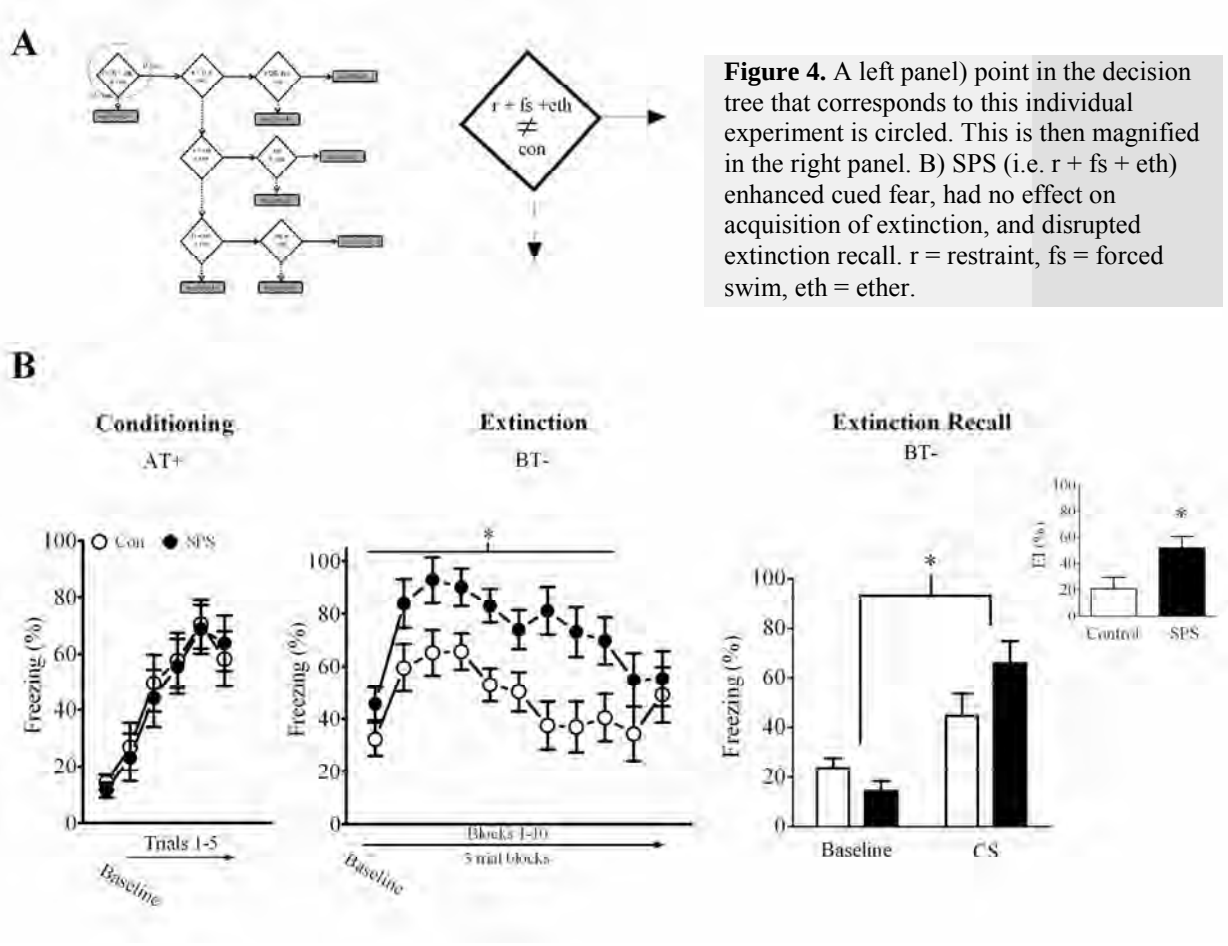


**Figure 4.** A left panel) point in the decision tree that corresponds to this individual experiment is circled. This is then magnified in the right panel. B) SPS (i.e. r + fs + eth) enhanced cued fear, had no effect on acquisition of extinction, and disrupted extinction recall. r = restraint, fs = forced swim, eth = ether.

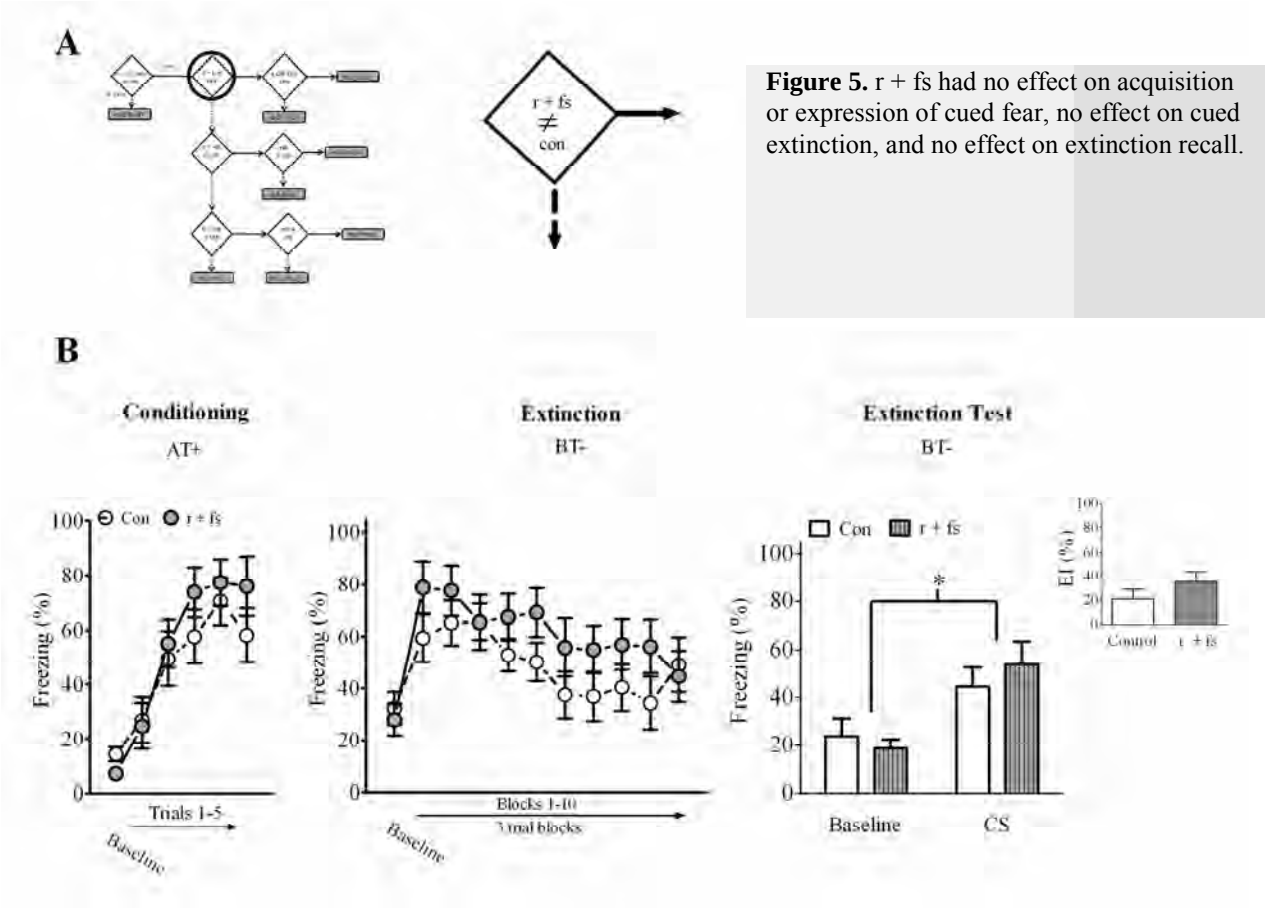


**Figure 5.** r + fs had no effect on acquisition or expression of cued fear, no effect on cued extinction, and no effect on extinction recall.

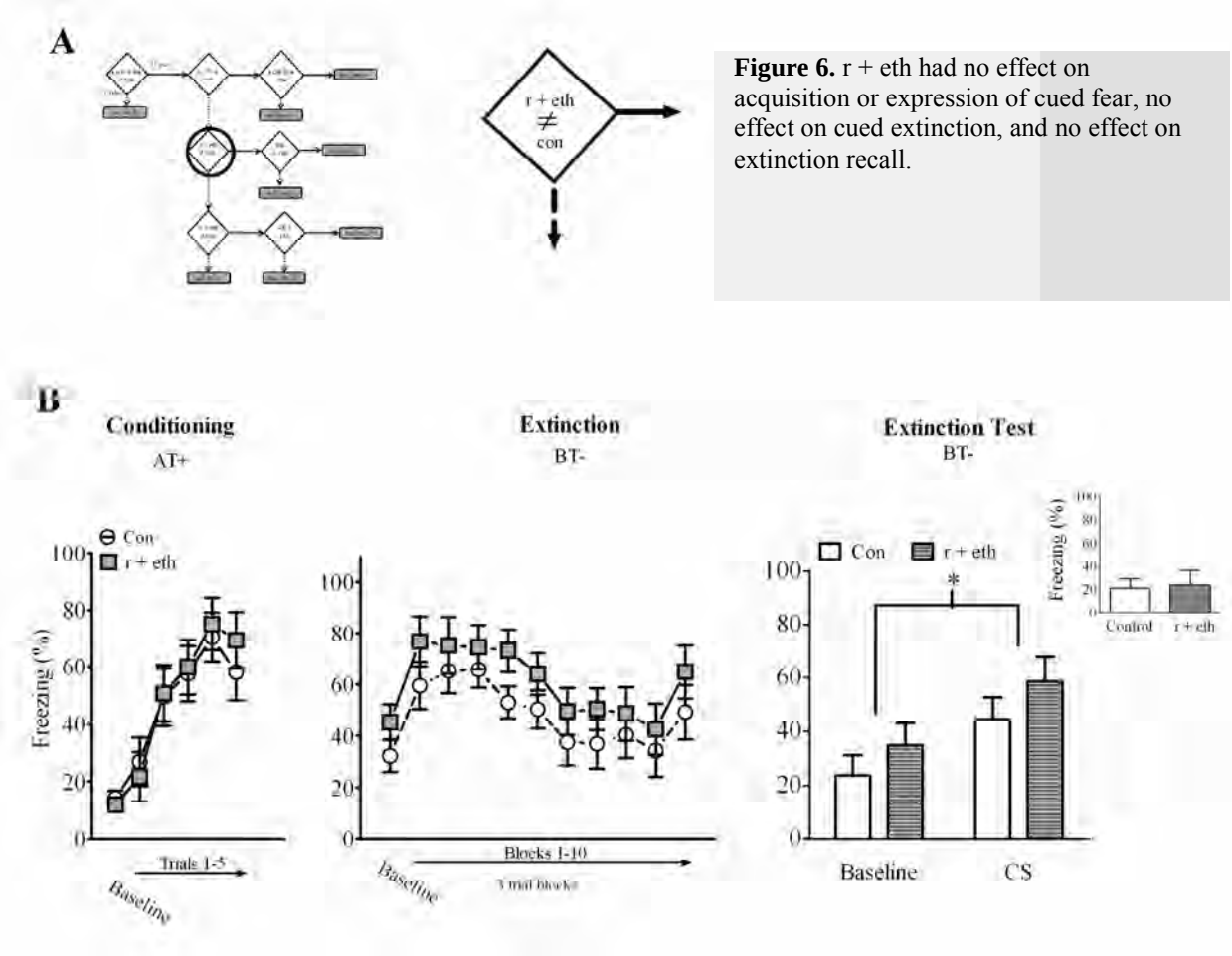


**Figure 6.** r + eth had no effect on acquisition or expression of cued fear, no effect on cued extinction, and no effect on extinction recall.

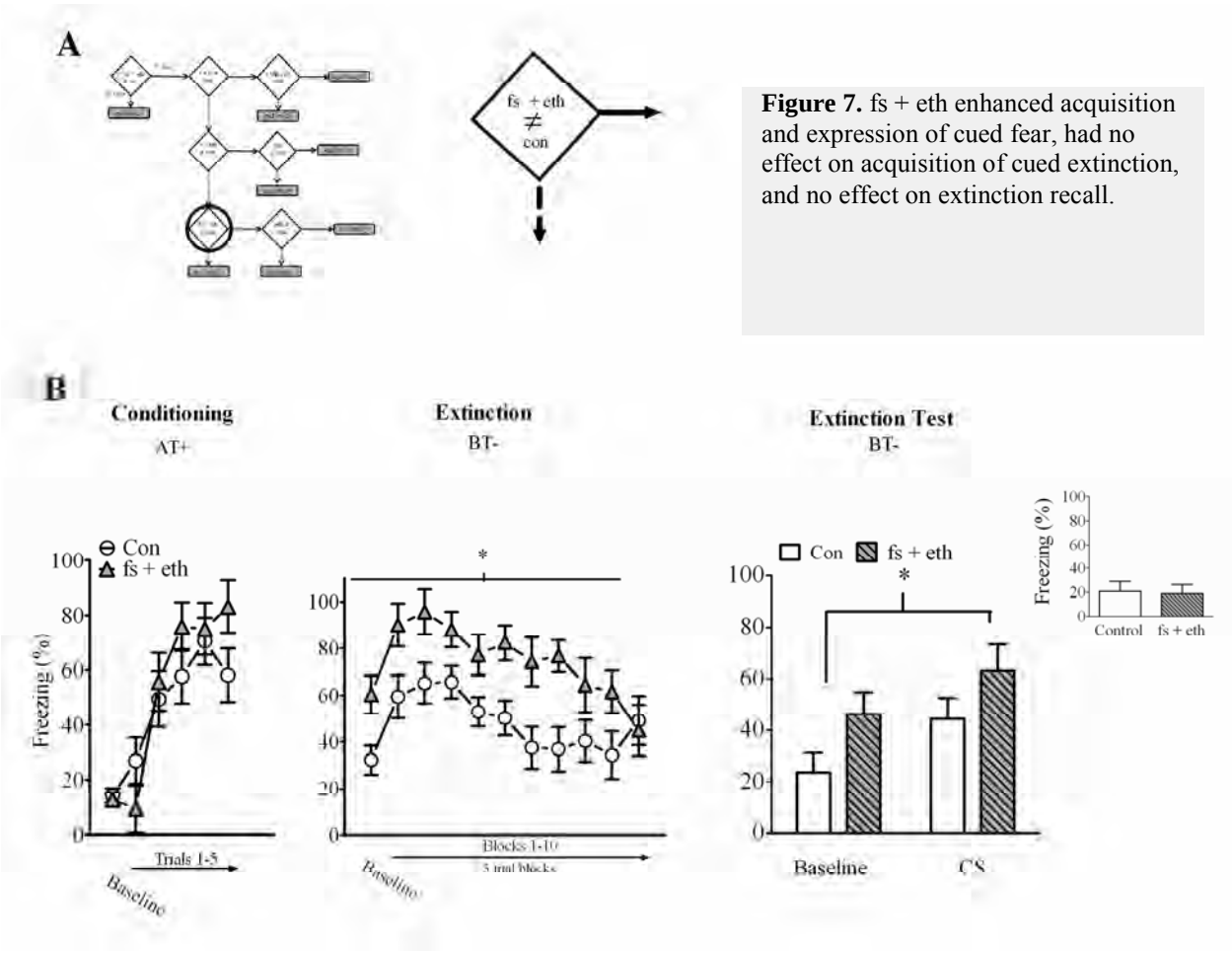


**Figure 7.** fs + eth enhanced acquisition and expression of cued fear, had no effect on acquisition of cued extinction, and no effect on extinction recall.

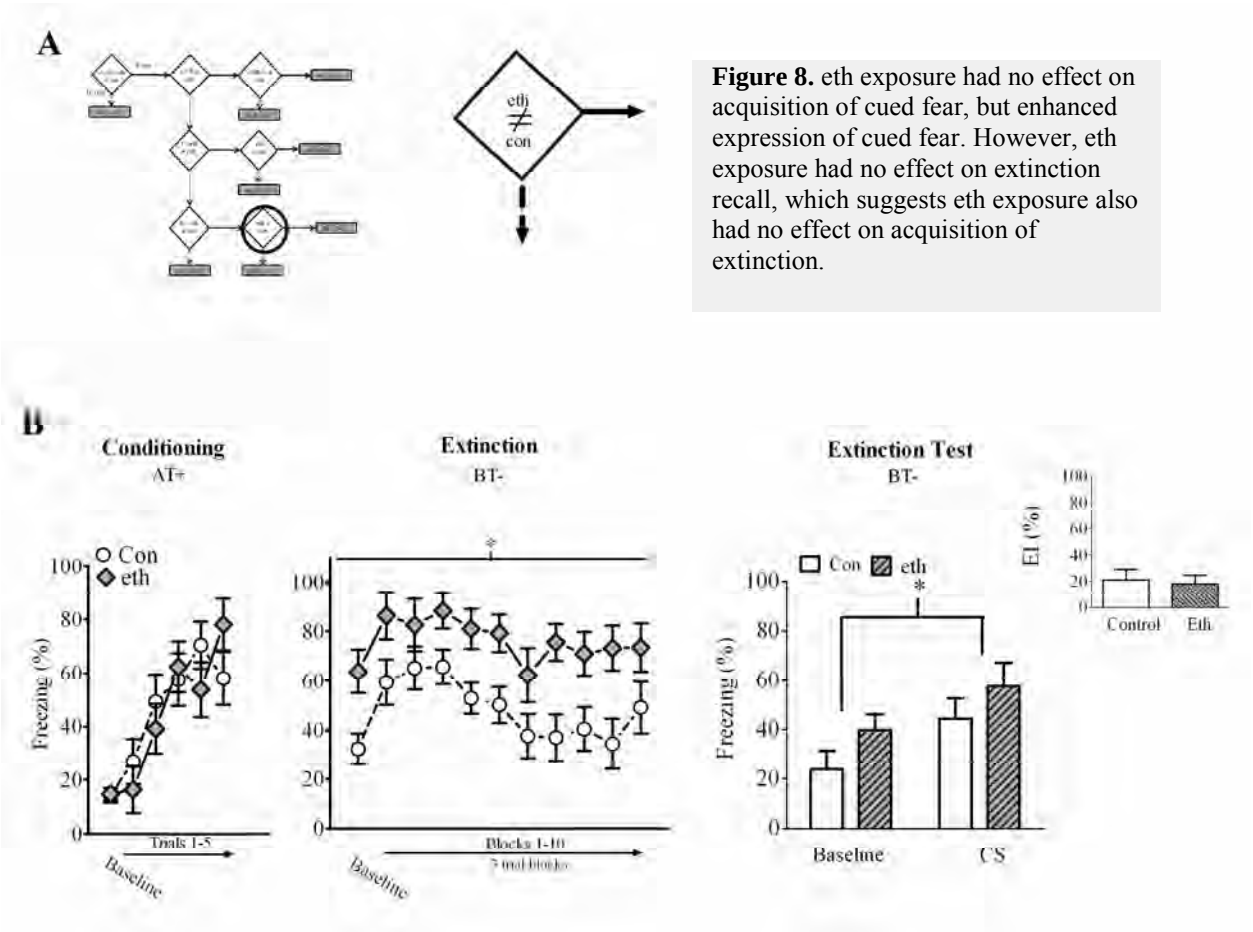


**Figure 8.** eth exposure had no effect on acquisition of cued fear, but enhanced expression of cued fear. However, eth exposure had no effect on extinction recall, which suggests eth exposure also had no effect on acquisition of extinction.

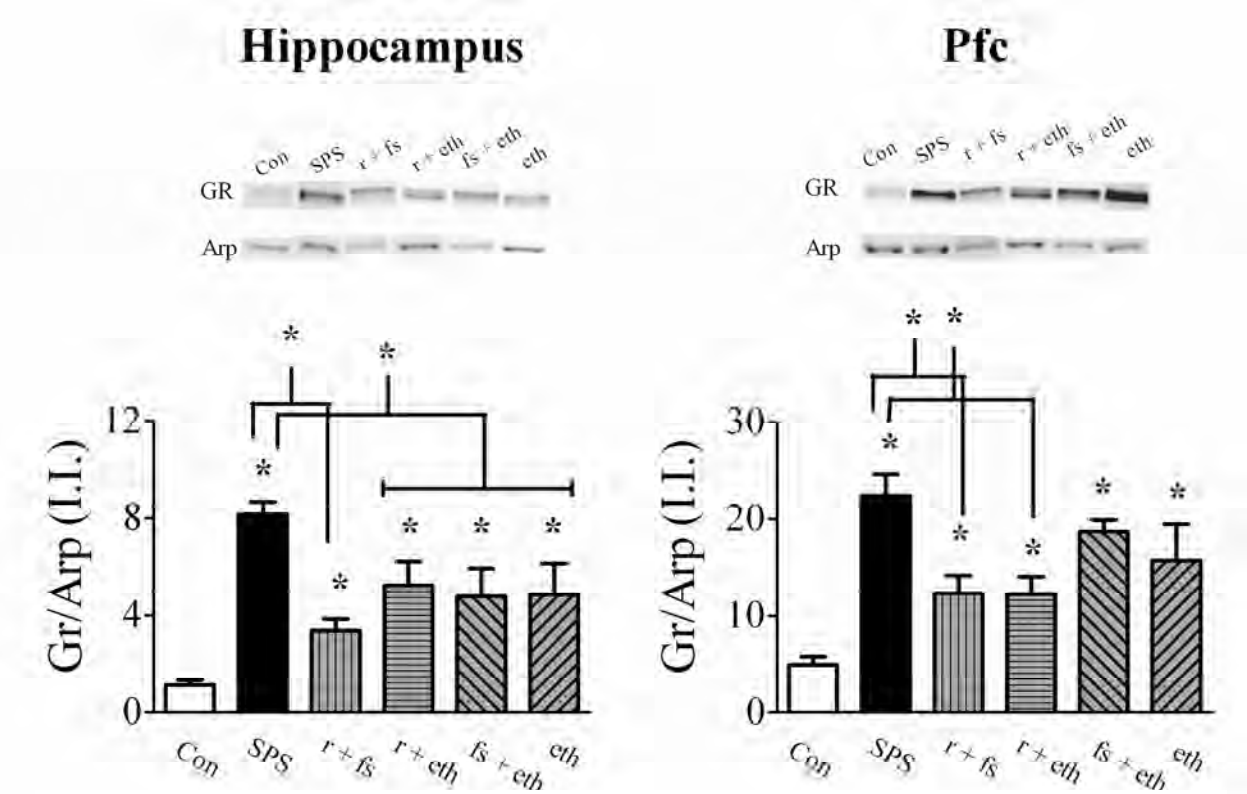


**Figure 9.** All stressors enhanced GR expression in the hippocampus and pfc, which is indicated by an asterisk over the bar. However, SPS produced the largest enhancement of GR in the hippocampus and pfc.

**3) Inactivation of the BLA attenuates social interactions**

In Specific Aim #2, our goal was to determine the role of the BLA in social interactions. The BLA is critical for expression of fear (Sotres-Bayon, Bush et al. 2004), and thus we hypothesized that inactivation of the BLA would enhance social interactions. In order to test this, we temporarily inactivated the BLA and examined the effect his had on social interactions using social interaction test (File and Seth 2003). As shown in Figure 10, BLA inactivation actually *decreased* social interactions. This suggests that the BLA may be critical for the generating or permitting social interactions, by for example, inhibiting anxiety during social encounters. In order to address this possibility, we conducted another experiment where we examined the effect of BLA inactivation on anxiety behavior in the elevated plus maze. In this test, BLA inactivation had no effect on anxiety behavior. This is illustrated in Figure 11. Thus, the results of this study suggest that the BLA may be involved in generating social behavior. We will explore the relevance of this finding to changes in social processes that are relevant to PTSD.

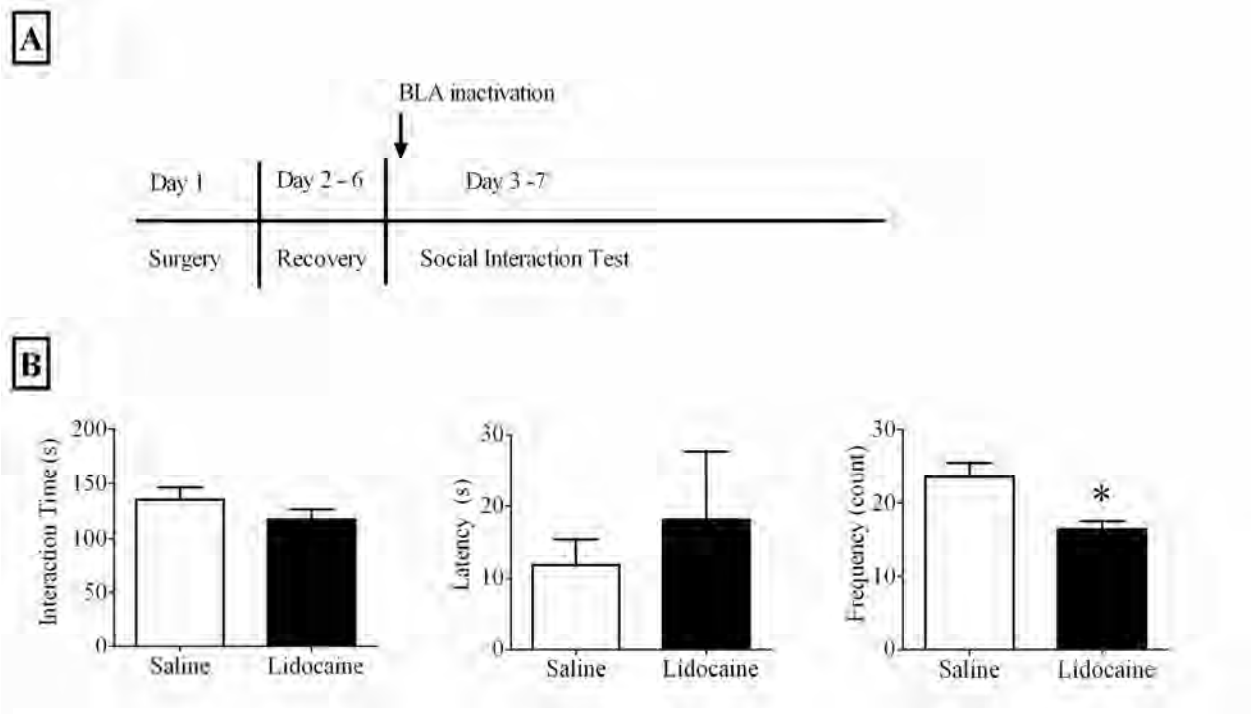


**Figure 10.** A) Experimental design for this study. B (left panel) Inactivation of the BLA had no effect on social interaction time, (middle panel) latency to the first social interaction, but (right panel) attenuated the number of interactions in the social interaction test.

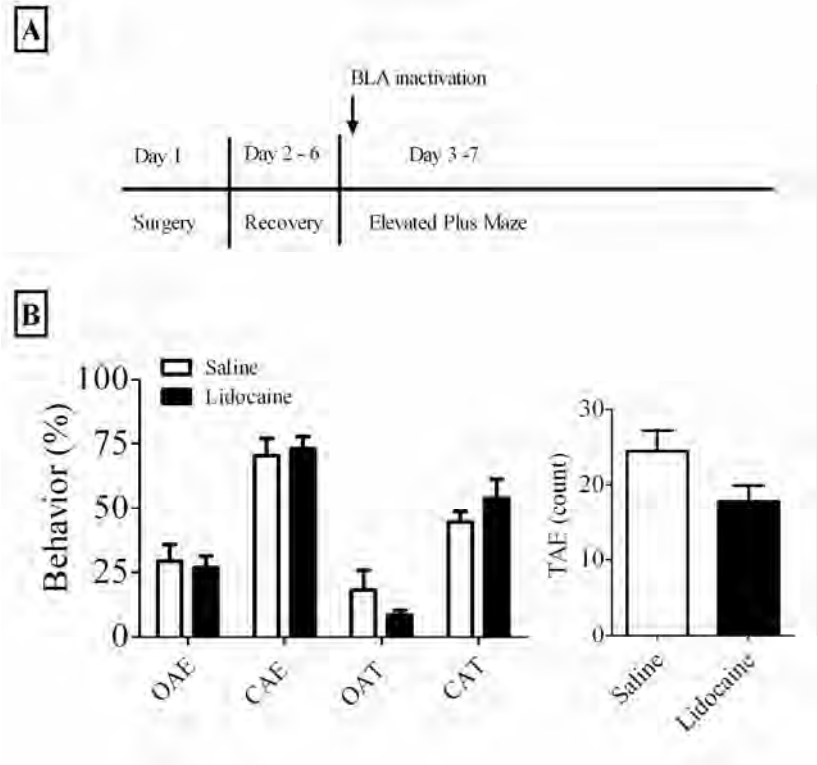


**Figure 11.** A) Experimental design for this study. B (left panel) Inactivation of the BLA had no effect on anxiety behavior or (right panel) total arm entries (locomotor activity metric) in the elevated plus maze. OAE – open arm entry, CAE – closed arm entry, OAT – open arm time, CAT – closed arm time, TAE – total arm entry.

**4) SPS enhances never reinforced errors during set shifting**

In specific aim #3 we were interested in determining the effects of SPS on emotional regulation. We originally planned to use predator odor-induced freezing paradigm (Specific Aim #3) to test the effects of SPS on emotional regulation, however the preliminary findings using this paradigm were not replicated, necessitating using an alternate approach to test emotional regulation. Our findings that SPS induces deficits in extinction recall reveal these deficit in emotional regulation (Quirk, Garcia et al. 2006). SPS rats fail to use extinction memories to regulate levels of fear. Similarly, our findings that SPS enhances fear renewal suggest that SPS-induced deficits in contextual processing disrupts extinction retention (Bouton, Westbrook et al. 2006). Thus, our extinction recall and fear renewal findings do suggest that SPS rats have a deficit in emotional regulation. Fear learning experiments alone however cannot identify all the specific cognitive mechanisms by which SPS exposure might lead in emotional regulation deficits. Emotional regulation, likely involves the use of a number of cognitive processes to regulate emotions (Campos, Frankel et al. 2004), thus given the inherent difficulties in setting up new experimental paradigms, we decided instead to directly examine the effects of SPS on other cognitive processes that may be of relevance to emotional regulation.

We examined the cognitive effects of SPS using reversal learning, occasion setting and set shifting experiments. The reversal learning and occasion setting experiment are still partially underway, thus we are focusing here on the results of set shifting. Set shifting is a form of behavioral and cognitive flexibility manifested in the animal's ability to learn a new strategy while inhibiting the execution of a previous strategy. For example, a rat can be trained to make a lever press in response to the position in which a cue light is illuminated (left or right). During the set-shift, the rat is required to ignore the cue light position, and instead press the lever according to its position in the chamber (on the left or right side). Deficits in set shifting have been implicated in PTSD (Walter, Palmieri et al. 2010), and may contribute to emotional regulation problems in this disorder (Walter, Palmieri et al. 2010).

As shown in Figure 12a, exposing rats to SPS did not affect learning of the initial rule, but impaired retrieval of this rule. SPS also did not disrupt the number of trials required to reach criterion on the set-shit task. However, when different types of errors were analyzed during set-shifting, SPS rats made more 'never reinforced errors', than controls. This suggests that SPS rats have a deficit in acquiring the new rule, such that they try more different kinds of behavioral strategies during the set-shift (Figure 12b). We will continue to explore this possibility as it pertains to emotional regulation in PTSD.

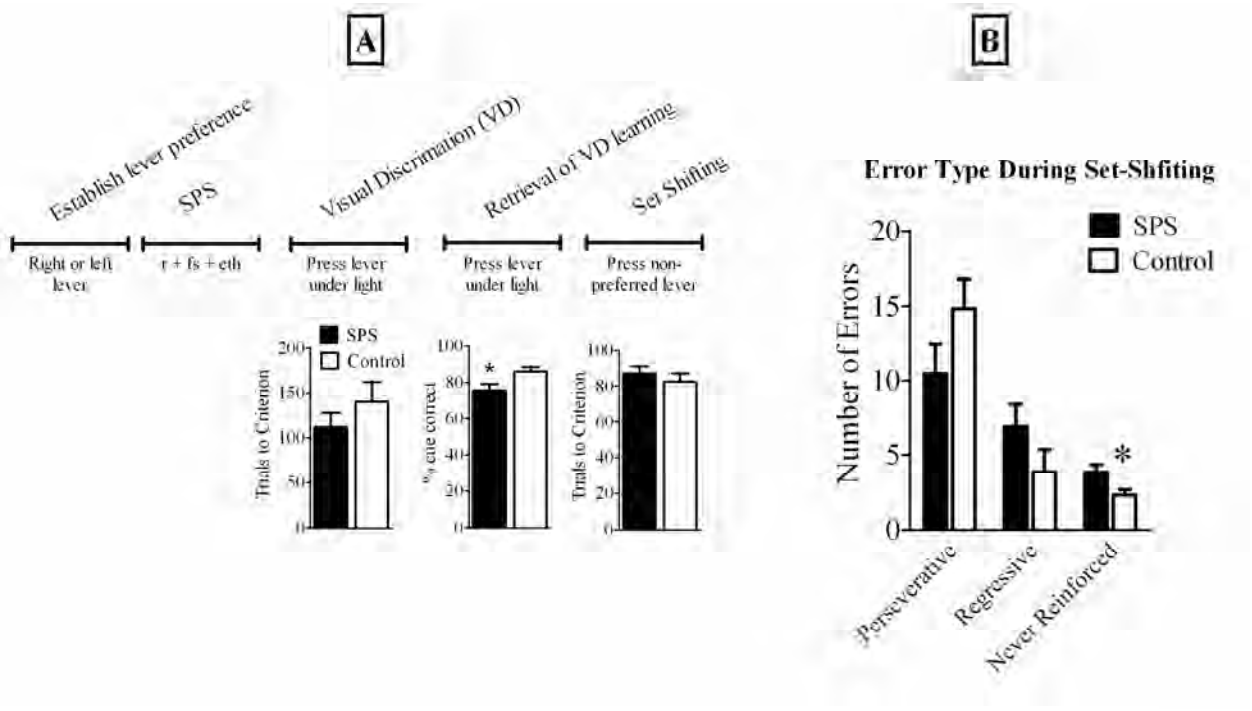


**Figure 12.** A) SPS had no effect on visual discrimination, but impaired memory retrieval of this discrimination rule. SPS did not disrupt set shifting, but B) increased the number of never reinforced errors during set shifting.

## Reportable Outcomes

The research we have conducted within the last year has resulted in two journal manuscript publications, two journal article submissions, four journal articles that are in preparation, and eight poster presentations. These are listed below.

### Journal articles

1) Knox, D., Perrine, S, George, S., Galloway, M., and Liberzon, I. (2010). Single Prolonged Stress Decreases Glutamate, Glutamine, and Creatine Concentrations In The Rat Medial Prefrontal Cortex. Neuroscience Letters, 480(1): 16 – 20. PMID: 20546834.

2) Fitzpatrick, C., Knox, D., Liberzon, I. (2011). Inactivation of the prelimbic cortex enhances freezing induced by trimethylthiazoline, a component of fox feces. Behavioural Brain Research: 22(1): 320 - 323. PMID:21420435.

3) Knox, D., George, S.A., Fitzpatrick, C.J., Rabinak, C., Maren, S., and Liberzon, I. (2011). Single prolonged stress disrupts retention of extinguished fear in rats. Learning & Memory: (submitted).

4) Knox, D., Stout, S., Tan, M., George, S.A., and Liberzon, I. (2011). Single prolonged stress enhancement of glucocorticoid receptor expression is partially attenuated by early handling. Stress: (submitted).

5) Knox, D., Tori, N., Henderson, C., and Liberzon, I. (2011). Identifying component of single prolonged stress that contribute to extinction retention deficits. (in preparation).

6) Knox, D., Fitzpatrick, C.J., George, S.A., Abelson, J.A. and Liberzon, I. (2011). Unconditioned fear is enhanced in an appetitive context. (in preparation).

7) George, S.A., Knox, D., Curtis, A., Valentino, R., and Liberzon, I. (2011). Altered noradrenergic activity in an animal model of PTSD. (in preparation).

8) Stout, S, Tan, M, George, S.A., Knox, D., and Liberzon, I. (2011). Are the effects of early handling on social interactions related to decreases anxiety or increased drive to socially interact? (in preparation).

### Conference proceedings

- 2011 Knox, D., Nault, T., Henderson, C., and Liberzon, I. Society for Neuroscience, Washington D.C. Linking single prolonged stress-induced extinction deficits to single prolonged stress enhanced glucocorticoid receptor expression in limbic regions.

- 2011 Fitzpatrick, C., Knox, D., George, S.A., and Liberzon, I. Society for Neuroscience, Washington D.C. Neural mechanisms by which SPS induces extinction retention deficits.
- 2011 George, S.A., Knox, D., Curtis, A., Valentino, R.J., and Liberzon, I. American College of Neuropsychopharmacology Annual Meeting, Waikaloa Beach, Hawaii. Altered noradrenergic activity in an animal model of post-traumatic stress disorder.
- 2011 George, SA., Knox, D., Curtis, A., Valentino, RJ., Liberzon, I. Altered noradrenergic activity following Single Pronged Stress, a rodent model of PTSD. Society for Biological Psychiatry Annual Meeting, 2011, San Francisco, CA.
- 2010 George, SA., Knox, D., Fitzpatrick, CJ., Abelson, JL., Liberzon., I. Chronic Phenytoin treatment reverses stress enhanced renewal, but not reinstatement, of conditioned fear in an animal model of post-traumatic stress disorder. American College of Neuropsychopharmacology Annual Meeting, 2010, Miami, FL.
- 2010 Knox, D., and Liberzon, I. Society for Neuroscience, San Diego. A comparison of the effects of TMT exposure and restraint stress on HPA axis function and noradrenergic systems.
- 2010 George, S.A., Knox, D., Fitzpatrick, C., Maren, S., Abelson, JL. Liberzon, I. Biological Psychiatry Annual Meeting, New Orleans LO. The effect of Single Prolonged Stress, a rodent model of PTSD, on extinction recall and reinstatement.
- 2010 George, S.A., Knox, D., Khan, S., Maren, S., Liberzon, I. Anxiety Disorders of America Association, Baltimore MD. The effect of Single Prolonged Stress, a rodent model of PTSD, on fear conditioning, extinction and extinction recall.
- 2010 Stout, S., Tan, M., George, S.A., Knox, D., Stern, E.R., Liberzon, I. Society for Neuroscience, San Diego. The effects of early life and adult stress on HPA-axis function and anxiety-like behavior.

## Conclusion

Based on the results of this research program we are able to conclude that 1) SPS-enhanced fear renewal is mediated by decreases in neural activity in the hippocampus and BLA, 2) SPS-enhanced GR expression in the hippocampus and PFC contributes to SPS-induced extinction recall deficits, 3) inactivation of the BLA attenuates social interactions, and 4) SPS enhances increases the frequency of a certain class of errors during set-shifting. What remains to be accomplished in the remainder of this research program is to a) further identify brain regions critical for mediating SPS-induced extinction recall deficits (Specific Aim #1), b) explore how the role of the BLA in facilitating social processes that are relevant to enhanced social avoidance in PTSD (Specific Aim #2), c) demonstrate how SPS-induced cognitive deficits might result in SPS-induced emotional regulation dysfunction (Specific Aim #3) and d) demonstrate that SSRI and antikindling drug treatment can reverse SPS effects (Specific Aim #4).

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Paxinos, G. and C. Watson (1998). The Rat Brain in Stereotaxic Coordinates. San Diego, Academic Press.

Quirk, G. J., R. Garcia, et al. (2006). "Prefrontal mechanisms in extinction of conditioned fear." Biol Psychiatry **60**(4): 337-343.

Sotres-Bayon, F., D. E. Bush, et al. (2004). "Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction." Learn Mem **11**(5): 525-535.

Walter, K. H., P. A. Palmieri, et al. (2010). "More than symptom reduction: changes in executive function over the course of PTSD treatment." J Trauma Stress **23**(2): 292-295.

## Supporting Data

Methods that were used to obtain the data presented in the Key Findings sections are presented here.

### Fear conditioning, extinction, extinction recall, and fear renewal

Behavioral Apparatus

All sessions were conducted in eight identical rodent observation chambers constructed of aluminum and Plexiglas (30 x 24 x 21 cm; MED Associates, St. Albans, VT), situated in sound-attenuating chambers and located in an isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center to center). The grid floor was connected to a shock source and a solid-state grid scrambler (MED Associates), which delivered the footshock unconditioned stimulus (UCS). Mounted on one wall of the chamber was a speaker to provide a distinct auditory conditioned stimulus (CS) on the opposite wall was a 15-W house light and a fan, which provided background noise (65 dB). Cameras mounted to the ceiling of the sound-attenuating chambers were used to record behavior, which was scored offline.

Two unique contexts were created by manipulating auditory, visual, and olfactory cues: Context A comprised a 1 % acetic acid solution placed in trays at the bottom of the chambers, the house light on, chamber doors closed, and fans on in the chambers; Context B comprised a 1% ammonium hydroxide solution in chambers, red light on, chamber doors open, and fans off.

Cued fear conditioning, extinction, and extinction recall

Rats were placed in Context A and received five paired presentations of a tone (10 s, 2 kHz, 80 dB) that co-terminated with a footshock (1.0 mA, 1 s) beginning 180 seconds after being placed in Context A. There was a 60 s inter-trial interval (ITI) and the rats remained in the chambers for 60 s after the last footshock presentation. One day after conditioning, all rats were placed into a novel context (Context B) and were presented with 30 tone presentations (10 s, 2 kHz, 80 dB, 60 s ITI), in the absence of footshock, beginning 180 s after being placed into the chambers in order to extinguish fear responding to the tone (i.e. extinction training). Two days after conditioning, rats were placed into the extinction context and were presented with 10 tones beginning 180 s after being placed into the chambers in order to assess extinction recall.

Data Analysis and Statistical Analysis

Videos from fear conditioning, extinction, and extinction recall were analyzed using Anymaze (Stoelting Inc., Kiel WI). This program uses changes in pixel contrast generated from movement in the video to create a movement score. Freezing is then determined by setting on and off thresholds that determine when freezing begins and ends, respectively. In this study, on and off thresholds were determined using a different data set to the one described in this study.

For fear conditioning, freezing values were averaged in trials that consisted of cues and respective ITIs. These scores were then analyzed using a two factors design with the first factor being stress (stress vs. control) and the second factor being fear conditioning session (baseline, trials 1-5). For fear extinction, freezing across three extinction trials were averaged into a block and analyzed using a two factors design with the first factor being stress and the second factor being fear extinction session (baseline, blocks 1-10). For extinction recall, freezing generated during the 10 trials was averaged into a single value and analyzed using a stress x extinction recall session (baseline, cue) factor design. Main and simple comparisons were analyzed using ANOVA, while main and simple comparisons were analyzed using t-tests, with a Bonferroni correction applied where necessary. The criterion for significance was set at $p < 0.05$. Rats that did not show a conditioned freezing response greater than 30 % at the start of a fear extinction session, were excluded from final analyses. All data are represented as means ± SEM.

### Detection of GR in the hippocampus and pfc using western blotting

One day after extinction recall all rats were removed from their housing colony and euthanized by rapid decapitation. Brains were removed, flash frozen in chilled isopentane, and stored in a -80 °C freezer until assay. In order to prepare brain homogenates, brains were thawed to -20 °C and the pfc dissected. The pfc was defined as all of the brain up until 2.7 mm anterior of bregma, with the exception of the olfactory tubercles. After removal of the pfc, the cerebrum was separated from the brain stem, thawed on ice, and the hippocampus was removed. Brain tissue was sonicated in homogenization buffer (50 mM Trizma base, 1 mM EDTA, 10 % Sucrose, 4 % sodium doedcyl sulfate, 2X protease inhibitor cocktail (Roche USA), pH 7.0 – 7.4), centrifuged at 105,000 x g,

homogenates decanted, and protein content determined using a Pierce BCA Protein Assay Kit (Sigma-Aldrich, St. Louis MO). Approximately 20 µg of protein was then diluted into a 1X Lamelli sample buffer and stored in a -80 °C freezer until western blot assay.

Assay of total GR protein levels (cytoplasm and nucleus) was adapted from Spencer and colleagues. Briefly, samples, along with a molecular weight (MW) ladder (LI-COR, Lincoln NE), were electrophoresed on 7.5% Tris HCl gels (Bio-Rad Laboratories, Inc., Hercules, CA ), transferred onto nitrocellulose membranes, and blocked in Tris buffer with 5% non-fat milk (i.e. blocking buffer (BB)). Membranes were then incubated with a rabbit polyclonal GR antibody (Santa Cruz biotechnology Inc., Santa Cruz CA, MR-20, 1:1000 in BB) overnight at 4 °C. After several washes in Tris buffer, membranes were incubated with an IRDye 800 conjugated anti-rabbit IgG secondary antibody (LI-COR, diluted 1:2000 in BB) for one hour, rinsed, and then scanned using the LI-COR Odyssey Scanner for visualization of GR bands.

Actin related protein (Arp) was used as the reference protein. Membranes were incubated with a rabbit polyclonal Arp antibody (Santa Cruz Antibodies, Arp-2, 1:2000 in BB), washed, and then incubated with an IRDye 800 conjugated anti-rabbit IgG secondary antibody (1:5000 in BB). Membranes were then rinsed and scanned in the LI-COR odyssey scanner in order to visualize Arp bands. We have previously used this method to detect GR levels in brain tissue.

Data Analysis and Statistical Analysis

Images of scanned membranes were analyzed using Odyssey software (LI-COR). The integrated intensity (I.I.) of the GR and Arp bands from stress and control samples were expressed as a ratio (GR/Arp) and used as a relative measure of GR levels. Relative hippocampal and pfc GR levels were subjected to t-test (stress vs. control). Main and simple effects were analyzed using ANOVA while main and simple comparisons were analyzed using t-test. Where appropriate, Bonferroni corrections were applied.

## Inactivation of the BLA

Prior to general anesthesia, rats were administered the muscle relaxant xylazine (Anased®, Ben Venue Laboratories, Bedford, OH; 20 mg/kg) subcutaneously (s.c.). General anesthesia was induced with 5 % isoflurane (Flurane®, Baxter Healthcare Corporation, Deerfield, IL) in oxygen flow, and maintained at 1-2 % isoflurane in oxygen flow during surgery. Depth of anesthesia was measured by the limb withdrawal and eye blink reflexes. No surgical procedures were conducted until both reflexes were absent. To prevent anesthesia-induced hypothermia, rats were warmed with a water-circulating heat pad (Gaymar Industries, Orchard Park, NY).

Stereotaxic surgery was performed by securing rats in a Kopf stereotaxic frame (Tujunga, CA), and rats were bilaterally implanted with stainless steel cannulae (26-gauge, 10mm; Plastics One, Roanoke, VA). The stereotaxic coordinates for cannulae implantation were as follows: BLA (A: -2.8 mm, L: ± 5 mm, D: -8.5 mm). All coordinates were referenced from bregma and based on the atlas of Paxinos and Watson (1998). Cannulae and three bone screws were bonded to the skull with dental acrylic. Dummy cannulae were inserted into the guide cannulae to maintain their viability. After surgery, rats were administered 3 mL of sterile saline s.c. to prevent dehydration. Experimentation continued when rats regained their pre-surgical body weight followed by two days of consecutive weight gain.

Temporary Inactivation.

The BLA was temporarily inactivated by infusion of the sodium channel blocker, lidocaine HCL (Sigma-Aldrich, St. Louis, MO) in a concentration of 2 %. Lidocaine HCl was dissolved in a .9 % saline solution, which was also used as the vehicle treatment. A microsyringe pump controller (Harvard Apparatus, Holliston, MA) with 5 µL syringes (Hamilton Company, Reno, NV) was used for infusions. Polyethylene tubing connected infusion cannulae (33-gauge, 11 mm; Plastics One, Roanoke, VA) to the syringes and controller. Solutions were infused bilaterally at a rate of 0.2 µL/minute for one minute and infusion cannulae remained in the guides for an additional minute. Behavioral procedures commenced 15 minutes later. Behavioral measures in the social interaction test and elevated plus maze were analyzed using t-test.

## Set-shifting

Apparatus

The behavioral apparatus consisted of eight operant boxes (Med Associates, VT, USA). Each box was housed in an individual chamber and was fitted with two Med Associates levers (VT, USA) situated on either side of a central magazine. The magazine was supplied by a pellet dispenser system (Med Associates, VT, USA), which delivered 45 mg food pellets (Test Diet AIN-76A Rodent Tablet, IN, USA). There were house lights located above

the central magazine to illuminate each chamber. In addition to the house light, two cue lights were located above each of the levers. The floor consisted of stainless steel rods. The boxes were connected to a computer using the MedPC software, capable of collecting output data from the boxes. The boxes were also connected to Med Associates interfaces, which controlled all experimental contingencies.

Procedure

After the rats were allowed to acclimatize for one week (while being food restricted as explained in detail in the Animals section), all rats were pre-trained for a lever press response in operant chambers. Initially all rats were placed in operant chambers and subjected to a fixed ratio-1 (FR-1) on one lever (counterbalanced left/right). After a rat reached criterion performance (>50 lever presses during a 30 minute session), it was placed back in its home cage. If a rat did not reach the criterion on the first day of training they received an additional identical training session the following day. Following this, rats' were trained on an FR-1, on the opposite lever until criterion was reached. For the next seven days rats were trained once daily on a time restricted FR schedule, during which one lever was presented every 20 seconds, and a response within 10 seconds resulted in delivery of a food reward. Each session included 90 trials, and criterion was reached after rats' exhibited less than fifteen omissions, defined as a trial during which a lever press response was not recorded within the 10 second period. If a rat reached criterion and had successfully completed a session with fifteen or less omissions before the full seven days had elapsed, then it was kept in its home cage for the remainder of this part of training. One day before SPS, all rats received one further session of training on the FR-restricted schedule. Rats were then matched on performance of the lever press response and assigned to SPS and control groups.

Following SPS, rats were placed in the operant chambers and trained on the time restricted FR procedure (described above) for three more days. On the last day of training, the side bias was determined for each rat. Side bias was determined by presenting the rat with both levers. On the first trial, a response on either lever resulted in pellet delivery followed by the retraction of both levers. After 20 seconds, both levers were extended again, but a reward was only given if a response was made on the opposite lever. This session consisted of seven trials and side bias was defined as either the lever which is pressed consistently more than the other (>60%), or the lever that is chosen initially.

The following day, the rats were again placed in an operant chamber, and a visual cue (the cue light) above one lever was activated three seconds before the levers were extended. A response on the lever corresponding to the illuminated cue light within 10 seconds constituted a correct response and pellet delivery. An incorrect response resulted in no reward and the end of the trial. Criterion performance was set at 10 consecutive correct trials, with a minimum of 30 trials completed. Once criterion was achieved, the program ended and rats were placed back in their home cages. If after 150 trials a rat had not reached criterion, a second day of training was repeated the following day.

Following acquisition of the visual cue rule, rats were trained on the set-shift task. For this session, the first 20 trials were identical to the visual cue training. For the remainder of the session the cue lights were still illuminated on the right or left side, but rats were rewarded for responding on the lever opposite to its side bias and had to ignore the cue light to make a correct response. After 10 consecutive correct responses, or after 180 trials had elapsed, the program ended and rats were placed back in their home cages. Rats that failed to reach criterion received a second day of training. Metrics on set-shifting involve perseverative errors, which can be defined as the inability to shift away from the original rule, regressive errors which refers to a tendency to go back to the original rule once the new rule has been acquired, and never reinforced errors which refers to errors that cannot be described as perseverative or regressive. Metrics in the set-shifting task were analyzed using a t-test.

# Appendix I

## Statement of work

**Institution name:** University of Michigan Medical School, 1500 Medical Center Dr., Ann Arbor, MI 48109;
Ann Arbor VA Healthcare Systems, 2215 Fuller Road, Ann Arbor, MI 48105.

**Personnel and effort:** Israel Liberzon MD, principal investigator (1.00 cal. months): Will oversee the whole project to ensure that all experiments are conducted in a timely fashion, consistent with the proposal, and in accordance with institutional guidelines and the principles of ethical use of animals in research. He will assure that all experiments are conducted with appropriate experimental rigor, and that data is published after completion of experiments. Dr. Liberzon will have primary responsibility for the interpretation of data and will primarily be responsible for writing research documents generated by this research proposal. Samir Khan PhD, co-investigator (6.00 cal. months): Will perform the bulk of experiments, data analysis, and research document preparation. Dayan Knox PhD, co-investigator (6.00 cal. months): Will perform the bulk of the experiments, data analysis, and research document preparation. Tony King PhD, co-investigator (0.60 cal. months): Will assist in developing protocols for protein and mRNA assays. Wayne Aldridge PhD, co-investigator (0.24 cal. months): will assist with electrophysiological experiments. TBA, research assistant (.60 cal. months years 1 & 2 and 6.00 cal. months years 3 & 4): will assist Drs. Khan and Knox in conducting all experiments. Stephen Maren PhD, consultant: will assist in electrophysiological and fear conditioning experiments.

**General Tasks (6/1/08 – 6/31/08):** 1) All equipment will be purchased within the first month of the release of funds to the University of Michigan. 2) All behavioral equipment will be purchased within the first month of the release of funds. 3) Electrophysiology equipment and molecular biology equipment required for testing hypotheses in specific aim 1 will be purchased within the first month of the release of funds to the University of Michigan.

**Experimental Animals:** Male Sprague Dawley rats will be used as subjects in all experiments. We anticipate an average of 15 rats/independent sample for all experiments in order to obtain statistical significance. However, this number will be adjusted from experiment to experiment used to test each hypothesis based on the difficulty of the experiments proposed, and the need for extra rats in the event that we are required to adjust the methods to deal with potential problems that may arise. In total we request 1,466 rats to complete the research proposal.

**Animal protocol:** All experiments will be staggered and a single animal protocol that includes all proposed experiments will be written in order to facilitate smooth transition from experiment to experiment. This protocol
will be written and submitted to the Veteran Affairs Institutional Animal Care Usage Committee within a month of notification that the University of Michigan has received the Intramural research award.

**Proposed experiments:** All experiments will be conducted between 6/1/08 – 5/31/12. Below we detail the experiments that we propose to conduct as they relate to each specific aim, and give time lines for the completion of these experiments. For all specific aims, the following sequence of tasks will be adhered to in order to allow for the execution of proper experimental protocols. A combination of temporary inactivation, single unit electrophysiology, pharmacological intervention, and molecular biology techniques will be used to test the hypotheses.

**Tasks:**

1) Purchase supplies for experiment (e.g. cannulas, electrodes, infusers, antibodies)
2) Purchase animals, perform SPS and/or drug procedures, and/or surgical procedures
3) Perform behavioral protocols (e.g. fear conditioning, extinction, social interaction)
4) Sacrifice rats and prepare tissue for histology or assay (e.g. Nissl stain, Western blot)
5) Perform assay (e.g. mRNA, protein), histology, or complete electrophysiology data analysis (within two weeks of termination of a particular experiment)
6) Repeat steps 2-5 at least once in order to replicate findings.

| **Specific Aim 1):** ***Examine the roles of altered mPFC function and expression of brain glucocorticoid receptors in the development of SPS induced extinction deficits (as a model of PTSD intrusive symptom cluster).*** | **No. of Animals** | **dates** |
|---|---|---|
| Hypothesis #1a: Temporary inactivation of the IL will lead to deficits in fear extinction in control rats, and this effect will be attenuated in SPS exposed rats. Methods used – cannula infusion, single prolonged stress, and fear conditioning | 75 | 6/1/11 – 10/30/11 |
| Hypothesis #1b: SPS exposure induces extinction deficits by altering neural activity in the IL. Methods used - Single unit electrophysiology, single prolonged stress and fear conditioning | 45 | 1/11/11 – 5/31/11 (partially completed) |
| Hypothesis 1c: SPS exposure induces extinction deficits by altering brain glucocorticoid receptor expression. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress. | 90 | 8/1/10 – 5/31/11 (completed) |

| **Specific Aim 2):** ***Examine the roles of altered mPFC/amygdala function in social interactions (as a model of PTSD social avoidance cluster), determine if social interactions can modulate SPS-induced changes in fear behaviors and HPA axis responses, and determine the importance of mPFC/amygdala activity and HPA/glucocorticoid receptor function in these SPS effects.*** | **Number of Animals** | **dates** |
|---|---|---|
| Hypothesis #2a: Temporary inactivation of the IL will lead to avoidance of social interactions. Methods used – cannula infusion, in situ hybridization, and social interaction test. | 75 | 6/15/11 – 8/31/11 |

| | | |
|---|---|---|
| Hypothesis #2b: Temporary inactivation of the BLA will increase social interactions. Methods used – cannula infusion, in situ hybridization, and social interaction test. | 75 | 6/15/11 – 9/30/11 (completed) |
| Hypothesis #2c: Social buffering will not attenuate SPS-induced changes fear and stress reactivity. Methods used - Single prolonged stress, brief maternal separation, western blot electrophoresis, startle reactivity, fear conditioning | 45 | 12/1/09 – 11/30/11 (completed) |
| Hypothesis #2d: Resistance to the beneficial effects of social buffering in SPS rats are due to aberrant neural activity in mPFC/amygdala circuits, and SPS-induced changes in the HPA axis. Methods used - Single prolonged stress, western blot electrophoresis, fear conditioning, in situ hybridization | 90 | 10/1/10 – 8/31/11 (partially completed) |

| **Specific Aim 3)**: ***Examine the role of altered mPFC/amygdala function and of altered HPA/glucocorticoid function in TMT-induced freezing, and determine if SPS disrupts social buffering of TMT-induced responses (as a model of emotional dysfunction in PTSD).*** | **Number of Animals** | **dates** |
|---|---|---|
| Hypothesis #3a: Temporary inactivation of the IL will lead to deficits in defense behavior regulation similar to that observed in SPS animals. Methods used – cannula infusion, single prolonged stress, and predator induced freezing | 75 | 8/1/10 – 10/31/10 (completed) |
| Hypothesis #3b: Temporary inactivation of the BLA will attenuate the defense behavior regulation deficit induced by SPS. Methods used – cannula infusion, single prolonged stress, and predator induced freezing | 75 | 11/1/10 – 01/31/11 |
| Hypothesis #3c: SPS exposure induces deficits in regulation of defensive behavior by altering neural activity in the IL/BLA. C-fos in situ hybridization, single prolonged stress and predator induced freezing | 45 | 9/1/11- 12/15/11 (completed) |
| Hypothesis #3d: SPS induced changes in defense behavior regulation are mediated, in part, by altered HPA axis/glucocorticoid function. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress. | 90 | 5/1/11- 7/31/11 |

| **Specific Aim 4):** ***Examine the ability of SSRI and antikindling drug administration to alleviate SPS induced extinction deficit and social buffering deficit; and the role of mPFC/amygdala activity, and HPA/glucocorticoid function in these processes.*** | **Number of Animals** | **dates** |
|---|---|---|

| | | |
|---|---|---|
| Hypothesis #4a. SSRI administration will attenuate SPS-induced extinction deficits and social buffering deficits by altering IL/BLA electrophysiological activity in SPS animals and by reversing changes in glucocorticoid receptor and mRNA expression in the prefrontal cortex, hippocampus, and hypothalamus. Methods used - Single unit electrophysiology, single prolonged stress, fear conditioning, social interaction, predator induced freezing, western blotting, in situ hybridization, and reverse transcriptase polymerase chain reaction. | 359 | 8/1/11 – 1/31/12 |
| Hypothesis# 4b. Antikindling/mood stabilizer administration will attenuate SPS induced defensive behavior regulation deficits by modulating neural activity in the IL/BLA. (two different drugs will be tested). Methods used - Single unit electrophysiology, single prolonged stress, and predator induced freezing. | 327 | 2/1/12 – 5/31/12 (partially completed) |

## Appendix II

**Definition of terms**

1. Fear Conditioning – Association of a neural stimulus (e.g. tone) with an aversive event (e.g. footshock).
2. Fear Extinction – The process of learning that a former fear conditioned stimulus no longer predicts an aversive event.
3. Extinction Recall – Remembering that a former fear conditioned stimulus no longer predicts an aversive event.
4. Fear Renewal – Extinction recall is optimal when tested in the context that extinction was learned. If extinction is tested outside of this context, then conditioned fear is observed. The return of conditioned fear when extinction is tested outside of the context in which extinction was learned is referred to as fear renewal.
5. Emotional Regulation – Processes that are critical for controlling emotional expression
6. Set-shifting - A form of behavioral and cognitive flexibility manifested in the animal's ability to learn a new strategy while inhibiting the execution of a previous strategy.
7. Social interaction test – This test is meant to examine social anxiety. Two stranger rats are placed in a context and the number of times they interact, times they spend interacting, or latency to the first interaction. Any change in any of these metrics is interpreted as a change in social interaction.
8. Elevated plus maze – This is a standard device used to measure anxiety behavior in rodents. It consists of a cross that has open arms and closed arms. The open and closed arms are perpendicular to each other. Rats tend to avoid the open arms (anxiety behavior) in preference for the closed arms. Also, total arm entries can be used as rough metric of locomotor activity.